Presented as an Honors Research Project to fulfill a requirement for graduation with distinction in Food Science and Technology.

Measurement and comparison of *Streptococcus thermophilius*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii* subsp. *shermanii* growth curves in synthetic medium and milk.

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Introduction

Humans have produced cheese, or a cheese-like product, since as early as 9000 years BC. The manufacture of Swiss cheese itself is centuries old. It requires the fermentation of milk by three different bacteria and the addition of rennet to aid in coagulation of milk proteins. Various strains of each bacterium are sold commercially to cheese-makers to ensure continuity of cheese quality and allow for variations in flavor, color, consistency, eye production and other properties (Kosikowski and Mistry, 1997).

In Swiss cheese making, mechanically clarified and heat-treated milk is inoculated with three starter cultures and rennet is added to coagulate the casein proteins in the milk. The curd is then cut and cooked (Kosikowski and Mistry, 1997). *Streptococcus thermophilus* grows in the milk during cooking by consuming lactose and producing lactic acid, thereby lowering the pH of the milk (Harrits and McCoy 1997).

Next, the curd is separated from the whey and pressed (Kosikowski and Mistry, 1997). *Lactobacillus helveticus* (or sometimes *Lactobacillus delbrueckii* subsp. *bulgaricus*) grows during the pressing of the cheese, as it starts to cool. This bacterium also converts lactose to lactic acid. The cheese is then soaked in brine (saturated NaCl solution) for 2-3 days. After brining, the cheeses are cooled to 10°C for about two weeks. During this time the lactose is depleted by *S. thermophilus* and *L. helveticus*. The resulting increase in acidity causes migration of calcium ions from the protein matrix and makes the cheese texture more elastic. The cheese is then transferred to the "warm room" (20 - 24°C) for 4 – 6 weeks where conditions are favorable for *Propionibacterium* growth. *Propionibacterium* converts the lactic acid produced by the other bacteria into propionic acid, acetic acid and carbon dioxide. The elastic texture of the cheese allows spherical eyes to form due to the carbon dioxide gas produced. Upon achieving the desired eye size and number, the cheese is transferred to the "cold room" (4 - 14°C) for 3 – 4 months where the propionibacteria should no longer grow or produce gas. Cold room storage allows bacterial proteolytic enzymes, primarily from *L. helveticus*, to partially breakdown the casein into peptides and amino acids. The texture of the cheese becomes firmer due the protein breakdown and colder temperature (Harrits and McCoy, 1997).

A problem observed in commercial production was that some *Lactobacillus* strains used in cheese manufacture had an adverse effect on the *Propionibacterium* fermentation causing little propionic acid formation and poor eye formation due to lack of CO_2. Other *Lactobacillus* strains have no effect on the *Propionibacterium* fermentation.

There are several complications in determining the cause of the problem. One complication in gathering information about starter cultures is that competing companies develop their strains and then keep their culture as a trade secret. Cheese producers select their strain combinations based on the manufacturers' recommendations and the reported characteristics of the strain. Characteristics commonly evaluated include rate of acid and carbon dioxide production. These characteristics are determined by growing the cultures in laboratory growth medium, not in milk. By growing cultures in milk, the characteristics of their role in cheese production will be more closely approximated. This information can be applied to selection of strains to produce better and more marketable cheese.

Materials and Methods

Origin of strains studied:

Starter cultures were obtained from three separate culture companies. Each culture was assigned an identification code. The first letter indicates the species, S for *S. thermophillus*, L for *L. helveticus*, and P for *P. freudenreichii* subsp. *shermanii*, three digit numbers were randomly assigned and cultures with an M following the three numbers are multiple strain cultures. The cultures were stored frozen until used. Single strain cultures were stored as glycerol stocks (20% glycerol), and multiple strain cultures were used from frozen pellets, frozen liquid, or frozen powder as obtained from the manufacturer. Many strains came from the culture companies with descriptions; these are summarized in Table A.

Growth curve parameters:

The three *Streptococcus thermophillus* strains were all single strain cultures. Frozen cultures were inoculated into L-M17, M-17 broth prepared according to manufacturer's directions (Difco, Detroit, MI) with the addition of 0.5% lactose. These cultures were incubated overnight at 42 °C and inoculated into fresh L-M17 the next day where it was again allowed to grow overnight. From the second overnight culture, a 1% inoculum was used to begin the growth curve. Growth curve medium was either L-M17 or UHT (ultra high temperature-treated) milk. The *S. thermophilus* strains were sampled every half hour until absorbance (600 nm) and pH readings indicated growth had stopped. Colony forming units per milliliter (cfu/mL) were obtained by standard plate count methods. All *S. thermophilus* strains were plated on L-M17 plates and counted after 24-48 hours of incubation at 42°C under aerobic conditions.

Single strain *Lactobacillus helveticus* cultures were prepared similarly to the *S. thermophillus* strains, except incubation was at 37 °C in an anaerobic chamber (85% nitrogen, 10% hydrogen, 5% carbon dioxide), and a 2% inoculum was used to start the growth curve. Samples were taken every hour until absorbance (600 nm) and pH readings indicated that growth had stopped. Broth and plates used for all *L. helveticus* strains were MRS medium prepared according to manufacturer's instructions (Difco, Detroit, MI). Multiple strain cultures were treated differently depending on their storage state. Strains that were shipped and stored as frozen pellets were divided into approximately 50 mL portions. To inoculate for a growth curve, one 50 mL aliquot was melted and mixed thoroughly. A 0.2% inoculation was made into growth curve medium. For the culture that was freeze-dried and stored as flakes, the flakes were dissolved into MRS for 4 hours to recover and then a 2% inoculation was made from the recovery broth. Frozen liquid cultures were thawed in the original container and a 0.2% inoculum was taken. Plates were incubated at 37°C in an anaerobic chamber for 36-72 hours.

Propionibacterium freudenreichii subsp. *shermanii* were inoculated from frozen cultures regardless of strain composition because single strain cultures failed to grow using the standard overnight culture inoculation procedure. The frozen culture was added to sodium lactate broth to approximate the optical density of an overnight culture. Sodium lactate medium was made with 1% tryptone, 1% yeast extract, 1% sodium

lactate, and 0.5% dibasic potassium phosphate and was standardized to a pH of 6.5 with sodium hydroxide. Diluted cultures were then inoculated into 200 mL of sodium lactate broth at a 0.2% inoculum. The broth was mixed and 10 mL aliquots were dispensed into sterile vials. The vials were capped with a rubber septum and crimp-top in an anaerobic chamber to achieve a low oxygen concentration and were incubated at 30°C. Samples were taken every four hours for 80 hours. At each sampling time, a vial was punctured with a needle attached to a gas measuring apparatus to determine gas production. The volume of gas produced was measured by water displacement in a graduated tube. The vial was then opened to measure absorbance at 600 nm, pH and cfu/ml. All *P. freudenreichii* subsp. *shermanii* strains were plated onto sodium lactate plates and incubated anaerobically at room temperature for a week. No *Propionibacterium* were grown in milk as research has shown that they do not grow well in unfermented milk due to their inability to anaerobically consume lactose (Fox, 2000).

Equipment used:

Absorbance was read at 600 nm in a Spectronic 20 spectrophotometer (Spectronic Instruments, Inc., Rochester, NY). A pH meter standardized at pH 4 and pH 7 was used to take pH readings on the samples. An anaerobic chamber (Forma Scientific, Marietta, OH) was used to grow samples in a limited oxygen environment.

Measurements and calculations:

Plates were made in duplicate over a range of tenfold dilutions to determine the number of colony forming units per milliliter (cfu/ml). Duplicate plates were averaged and the results were graphed. Absorbance and pH readings were taken once at each time point. The data were graphed using SigmaPlot 2000 (SPSS, Inc. Chicago). Regression analysis using the Gompertz equation, $y=y_0+ae^{-e(x-xo)/b}$, was applied to the data to determine the maximum rates and maximum or minimum values attained by the culture.

Results and Discussion

Streptococcus thermophilus:

The results of the *S. thermophilus* growth curves in broth and milk are reported in Tables 1 and 2 and Figures 1 through 5. In broth and in milk, S187 has the highest maximum growth rate. However, S884 exhibited the highest maximum cell density, the maximum rate of acidification of the medium, the greatest overall acidification and the lowest pH in broth. It also showed the highest maximum cell density and maximum rate of acidification in milk. The greatest overall change in pH and the lowest pH in milk were achieved by S692. The rank of final pH values in broth was exactly opposite the rank of final pH values in milk. These results illustrate that if strain performance is only tested in broth, the characteristics for which the strain is chosen may not be the same in milk. Since the production of cheese is dependent on the lowering of pH, S692 is recommended for rapid acid production in the milk. The average maximum growth rate of all strains in milk was faster than in broth, though broth had a greater maximum cell density. The pH reduction was much greater in broth and the maximum rate of acidification of the media was higher in the milk. The minimum pH values of the milk were lower than those of the broth, which may indicate that the growth is limited by nutrient depletion in the medium, not by pH limiting growth. Comparison of the change in pH is complicated by the different buffering capacity of each medium.

Lactobacillus helveticus:

The results of the *L. helveticus* growth curves in broth and milk are reported in Tables 3 and 4 and in Figures 6-10. There were not many similarities when comparing strain growth in broth with growth in milk. This further supports the importance of testing strains in milk, not broth. The ranking of maximum cell density of the strains in broth were similar to the ranking of maximum cell density values obtained in milk but were not the same numbers in the different media. Another difference noted is that the maximum rate of acidification for each strain was not significantly different in the broth, but there was a wider range in milk. Again, these cultures are employed to lower the pH of the cheese and produce lactic acid for the *Propionibacterium* to consume during their

growth. Therefore, the amount and rate of acid production are important. The data collected is inconclusive in determining more advantageous strains. Average maximum growth rates were faster and maximum cell densities were higher in broth than in milk. The average rate of acidification was faster in broth than in milk but the average change in pH was greater and the final pH was lower in milk than in broth.

***Propionibacterium freudenreichii* subsp. *shermanii*:**

The results of the *Propionibacterium freudenreichii* subsp. *shermanii* growth curves are reported in Table 5 and Figures 11-14. The strain that grew fastest in the medium, P307M, also produced gas at the fastest rate. Both of these values were much higher than the values for the other strains. The strain that grew to the highest maximum cell density had a low maximum growth rate and a low rate of gas production. Since gas production is important in the formation of the eyes in Swiss cheese, this criterion should be used to choose the strain. However, the production of propionic and acetic acids are also a consideration in strain selection. The pH was measured in the medium, but there was little change since these bacteria consume lactic acid, producing propionic and acetic acid.

Limitations and problems encountered:

The first problem encountered was variability in the number of cells initially inoculated into the media. Due to the variability in number and viability of the frozen cultures or the stationary phase cultures, it was impossible to inoculate the same number of organisms into each medium at the beginning of every growth curve.

Another problem that is possibly related to the first is the occasional failure of growth to follow established growth patterns. Normal growth is considered to have a lag phase where little growth occurs, an exponential phase in which the microorganism is doubling at maximum rates, a stationary phase where growth stops and a death phase. The lag phase was minimized in this experiment by using freshly grown overnight cultures where possible, by using a recovery time for freeze dried samples, and by using media that meet the nutrient requirements of the organisms grown. In cases where a normal growth pattern was not achieved, the data was not used. Most strains consistently

exhibited normal growth, while a few others required several attempts to produce the pattern. S187, L651, and L846M all required multiple attempts in broth and in milk. These strains showed only slight if any increase in number of cells and little or no decrease in pH over the course of the 9 or 15 hours they were monitored. The experimental causes of aberrant growth patterns were very low viable inoculum or contamination by other microorganisms. Industrially, four main causes contribute to starter failure, natural inhibitors in the milk, antibiotics in milk, bacteriophages and bacteriocins (Fox, 2000). However, since all milk used was from the same lot and high quality, UHT milk, these are likely not a concern. Culture reliability should be taken into account when selecting strains for use in cheese manufacture to decrease loss due to starter failure.

A problem encountered in the laboratory, but not new to the cheese industry, is the effect of background microflora in the milk. Initially, the growth curves were attempted in rehydrated non-fat dry milk that had steamed in an autoclave (100 °C for 10 minutes). This heat treatment was not adequate to inactivate all the microorganisms in the milk. Contamination was to the extent that the growth of the inoculated strain was undeterminable. The next milk trials were done in microfiltered milk obtained from Holmes Cheese Company, Millersburg, OH. This milk was considerably less contaminated, but still contained both *Streptococcus* and *Lactobacillus* strains in enough quantity to outcompete the starter strain. Finally, ultra high temperature (UHT) pasteurized milk was used. The UHT milk did not contain microorganisms able to grow under the conditions used in this study. It is also possible that some problems in industry are related to the starter culture strains' abilities to compete with the existing microflora in the milk.

Measuring gas production using crimp-top vials with rubber septa proved to be problematic and not very accurate. The initial readings seemed to be appropriate, with minor amounts of gas production. However, as the pressure continued to increase, the values became more sporadic. Also some vials actually pulled a vacuum when attached to the measuring apparatus. The vials are likely incapable of providing a perfect seal at the pressures reached for the duration of the experiment. The cause of the vacuum is unknown at this point. At a few time points, the gas production for all strains was

consistently low. This flaw could be related to the fit between the needle and the gas apparatus. Lastly, the vials were capped in a controlled atmosphere with variable pressure. This means that there was variation in the starting pressures in the vials and therefore inaccuracy in the amount of gas produced. Without major improvements, this method of gas production measurement is not recommended for this application.

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Conclusions

1. Commercial starter cultures of *S. thermophilus*, *L. helveticus* and *P. freundenreichii* vary in their growth rates and rates of acid and carbon dioxide production.
2. Growth and acid production rates of a particular strain differ in milk and laboratory medium. When selecting starter cultures for industrial use, the strains should be evaluated in medium similar to the food production medium.
3. Growth rate was not indicative of acidification rate or overall pH change in *S. thermophilus* and *L. helveticus* or of gas production rate in *P. freudenreichii*.
4. This experiment confirmed that the absorbance (600 nm) of a culture correlates with the number of cells. Culture absorbances cannot be compared across strains or species.

Acknowledgements

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References

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2 Harrits, J. and D. McCoy. 1997, Swiss cheese. In *Cultures for the Manufacture of Dairy Products.* CHR Hansen, Milwaukee, WI

3 Kosikowski, F.V., and V. V. Mistry. 1997, Cheese with Eyes. In Cheese and Fermented Milk Foods. F.V. Kosikowski, L.L.C. Westport, CT,

Table A: Strain sources and descriptions.

Strain designation	Source[a]	Description of acid production
S884	Company A	Fast
S692	Company B	Slow
S187	Company C	Fast
L879	Company A	Information not provided
L578M	Company A	Medium
L846M	Company B	Medium
L267	Company B	Medium
L450	Company B	Medium
L966	Company B	Medium
L143	Company B	Medium
L651	Company B	Medium
L201	Company C	Slow
L374M	Company C	Slow
P159	Company A	Information not provided
P791	Company A	Information not provided
P518	Company B	Information not provided
P947M	Company C	Information not provided
P307M	Company C	Information not provided
P664M	Company C	Information not provided

[a] Companies wished to remain anonymous to maintain trade secrets.

Table 1: Growth and pH parameters for *S. thermophilus* strains grown in L-M17 broth for 9 hours.

Strain	Maximum growth rate [(log cfu/ml)/hour]	Maximum cell density (log cfu/ml)	Maximum rate of acidification (pH/hour)	Overall change in pH	Final pH
S884	0.68	8.51	-0.85	-2.67	6.64
S692	0.67	8.19	-0.70	-2.28	6.98
S187	1.56	8.33	-0.70	-1.95	6.68

Table 2: Growth and pH parameters for *S. thermophilus* strains grown in milk for 9 hours.

Strain	Maximum growth rate [(log cfu/ml)/hour]	Maximum cell density (log cfu/ml)	Maximum rate of acidification (pH/hour)	Overall change in pH	Final pH
S884	0.85	8.44	-2.88	-0.36	6.22
S692	0.88	7.79	-0.71	-1.16	5.43
S187	1.61	8.43	-0.50	-0.75	5.98

Table 3: Growth and pH parameters for *L. helveticus* strains grown in MRS broth for 15 hours.

Strain	Maximum growth rate [(log cfu/ml)/hour]	Maximum cell density (log cfu/ml)	Maximum rate of acidification (pH/hour)	Overall change in pH	Final pH
L267	0.33	8.64	-0.32	-2.12	4.39
L450	0.43	8.63	-0.31	-2.06	4.43
L966	0.53	8.89	-0.32	-2.03	4.39
L143	0.37	9.23	-0.29	-2.41	4.14
L651	0.24	9.35	-0.33	-1.98	4.36
L201	0.40	9.12	-0.34	-2.51	4.26
L879	0.43	9.08	-0.33	-1.75	4.51
L374M	0.40	8.93	-0.34	-2.06	4.40
L578M	0.36	8.62	-0.32	-2.34	4.24
L846M	0.30	8.63	nd	nd	nd

Table 4: Growth and pH parameters for *L. helveticus* strains grown in milk for 15 hours.

Strain	Maximum growth rate [(log cfu/ml)/hour]	Maximum cell density (log cfu/ml)	Maximum rate of acidification (pH/hour)	Overall change in pH	Final pH
L267	0.39	8.96	-0.38	-2.77	3.89
L450	0.36	8.41	-0.36	-2.70	3.99
L966	0.32	9.21	-0.29	-2.94	3.89
L143	0.57	9.11	-0.27	-2.88	3.91
L651	0.21	9.10	-0.28	-2.86	3.88
L201	0.18	9.03	-0.24	-2.90	3.90
L879	0.15	8.97	-0.28	-2.69	3.95
L374M	0.24	8.70	-0.29	-2.74	4.00
L578M	0.38	8.63	-0.23	-3.35	3.89
L846M	0.26	8.75	nd	nd	nd

Table 5: Growth and gas production parameters for *Propionibacterium* strains grown in sodium lactate broth for 80 hours.

Strain	Maximum growth rate [(log cfu/ml)/hour]	Maximum cell density (log cfu/ml)	Maximum gas production rate (ml/hour)
P159	0.058	9.84	0.013
P791	0.086	9.46	0.017
P518	0.18	9.44	0.055
P947M	0.050	9.76	0.057
P307M	0.45	9.48	0.11
P664M	0.065	9.53	0.058

Figure 1: Growth of three *Streptococcus thermophilus* strains in L-M17 broth

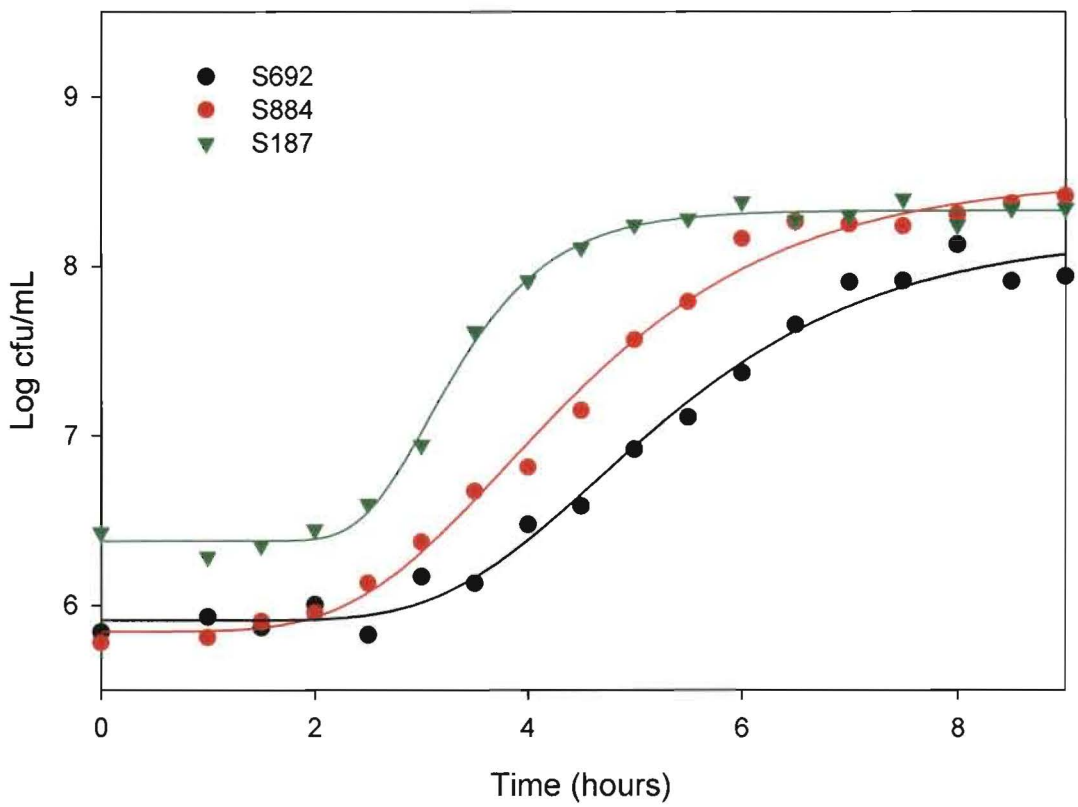

Figure 2: pH of three *Streptococcus thermophilus* cultures in L-M17 broth

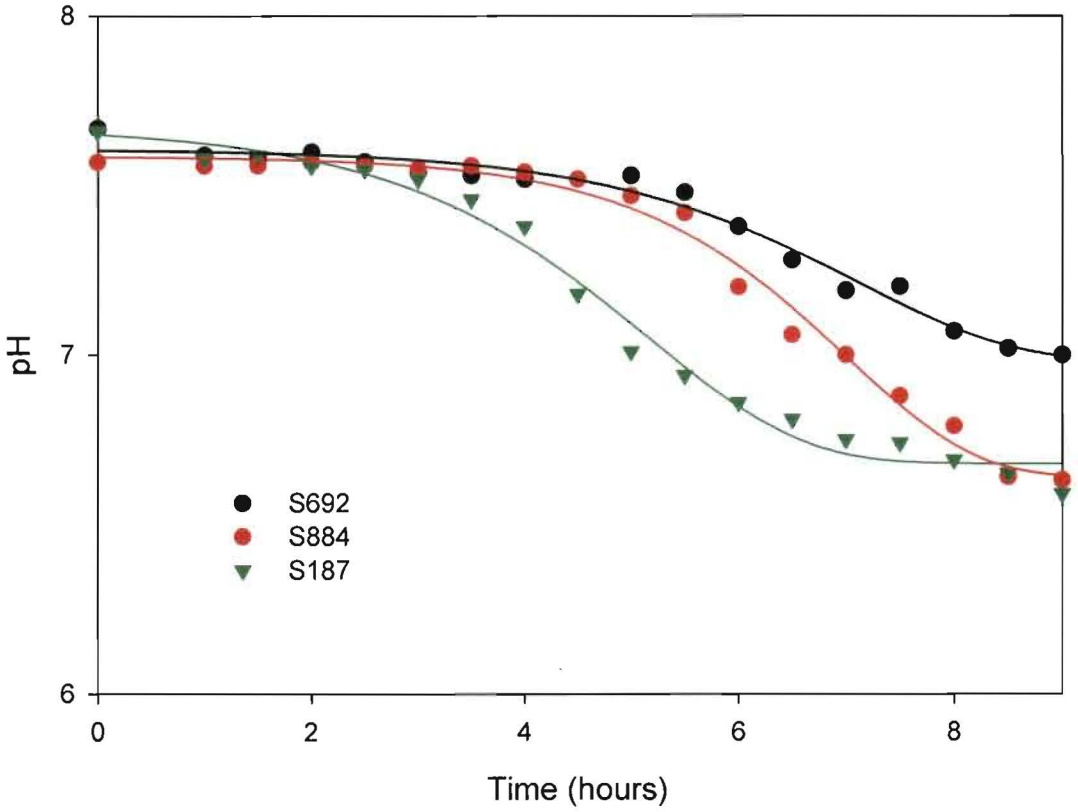

Figure 3: Absorbance (600 nm) of three *Streptococcus thermophilus* cultures in L-M17 broth.

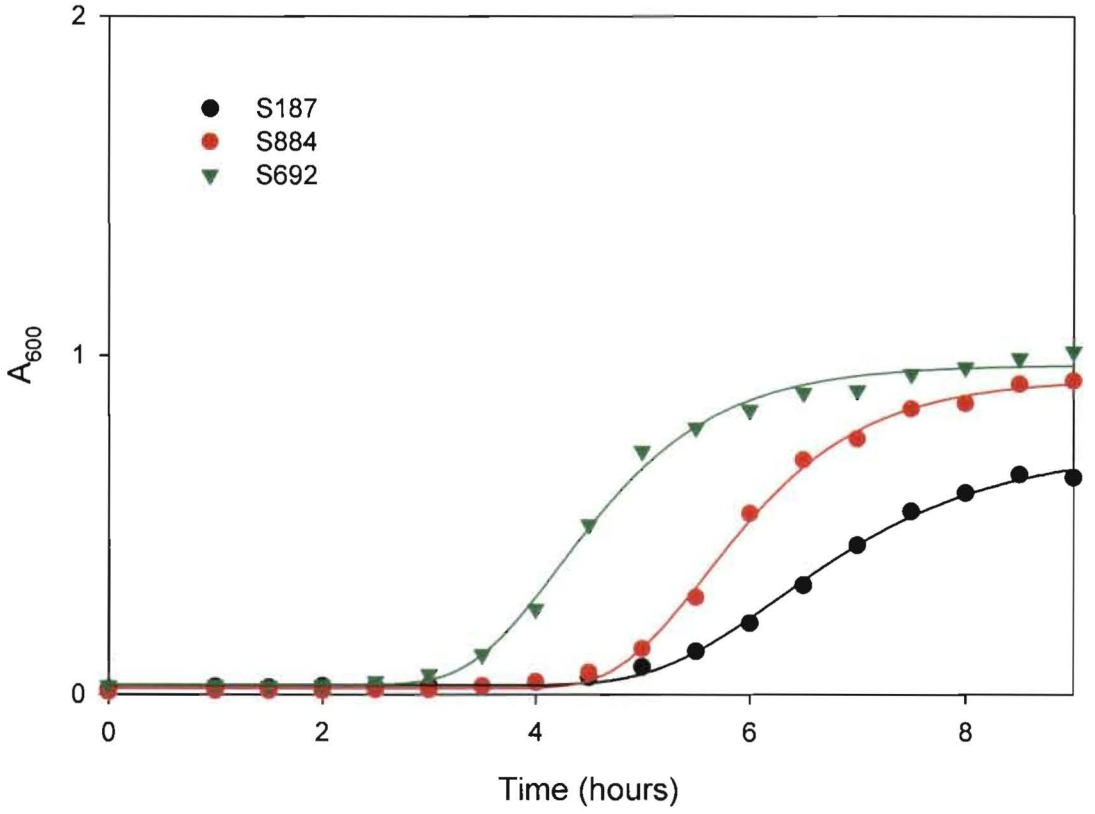

Figure 4: Growth of three *Streptococcus thermophilus* strains in milk.

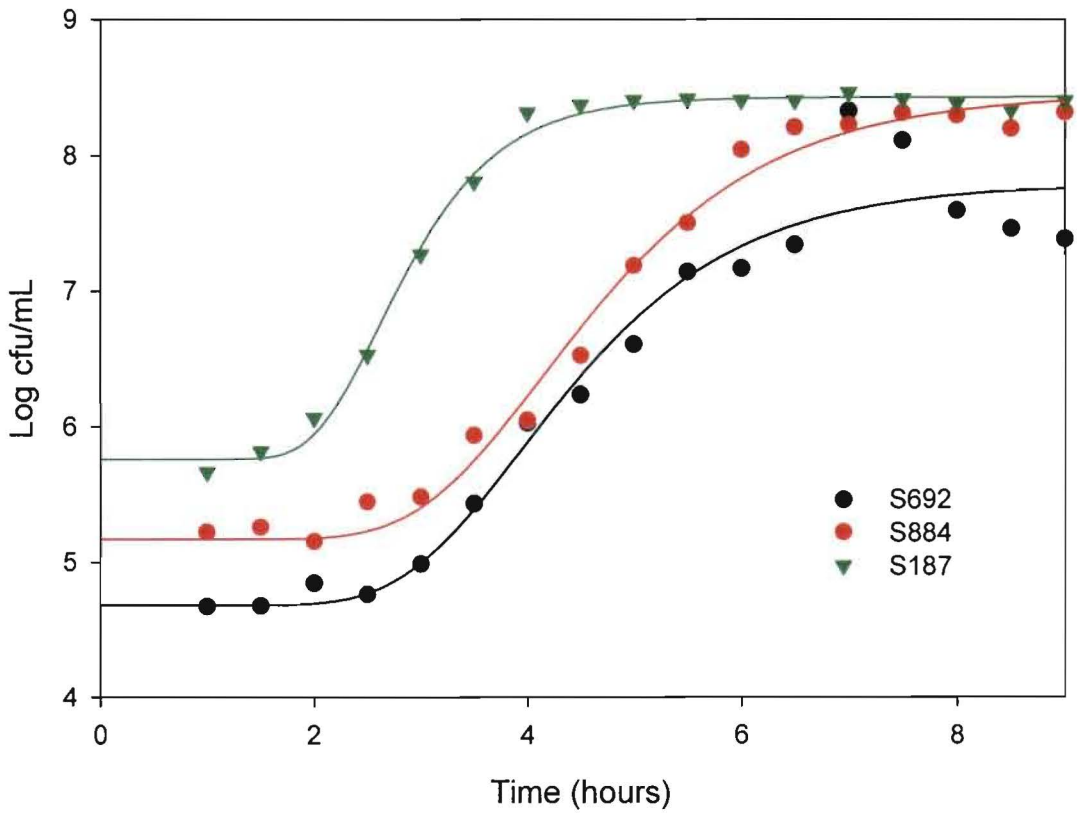

Figure 5: pH of three strains of *Streptococcus thermophilus* cultures in milk

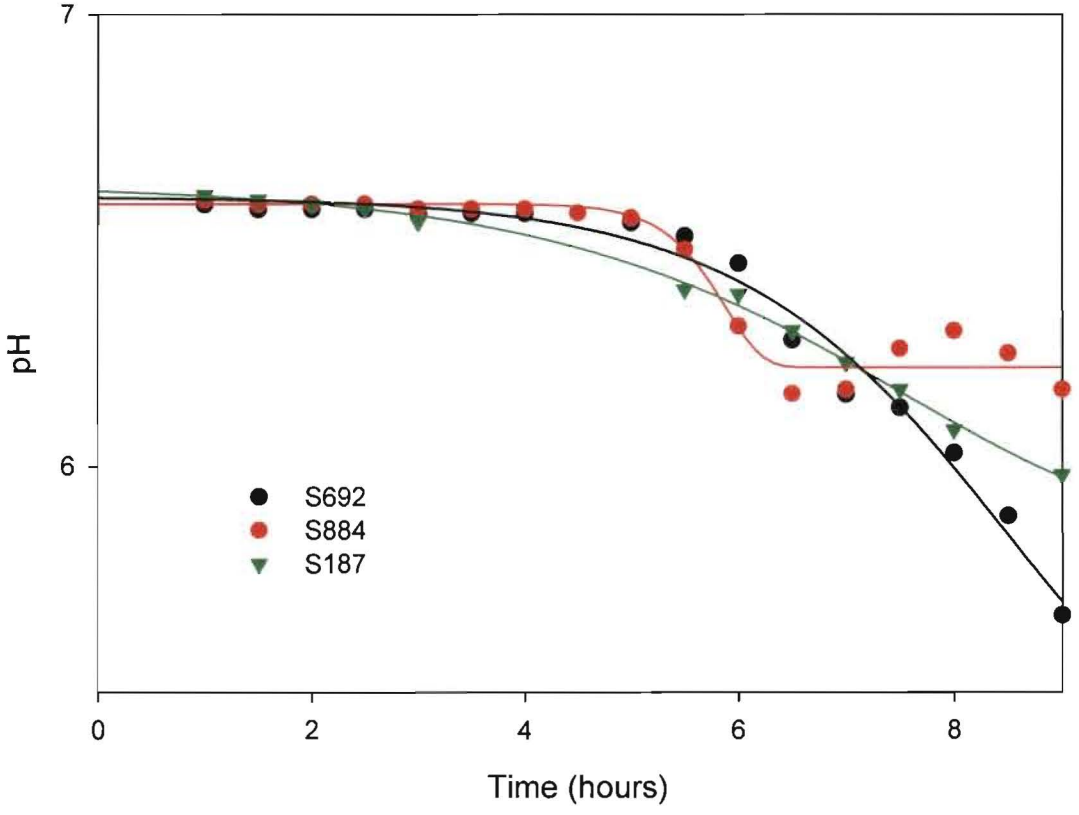

Figure 6: Growth of ten *Lactobacillus helveticus* strains in MRS broth.

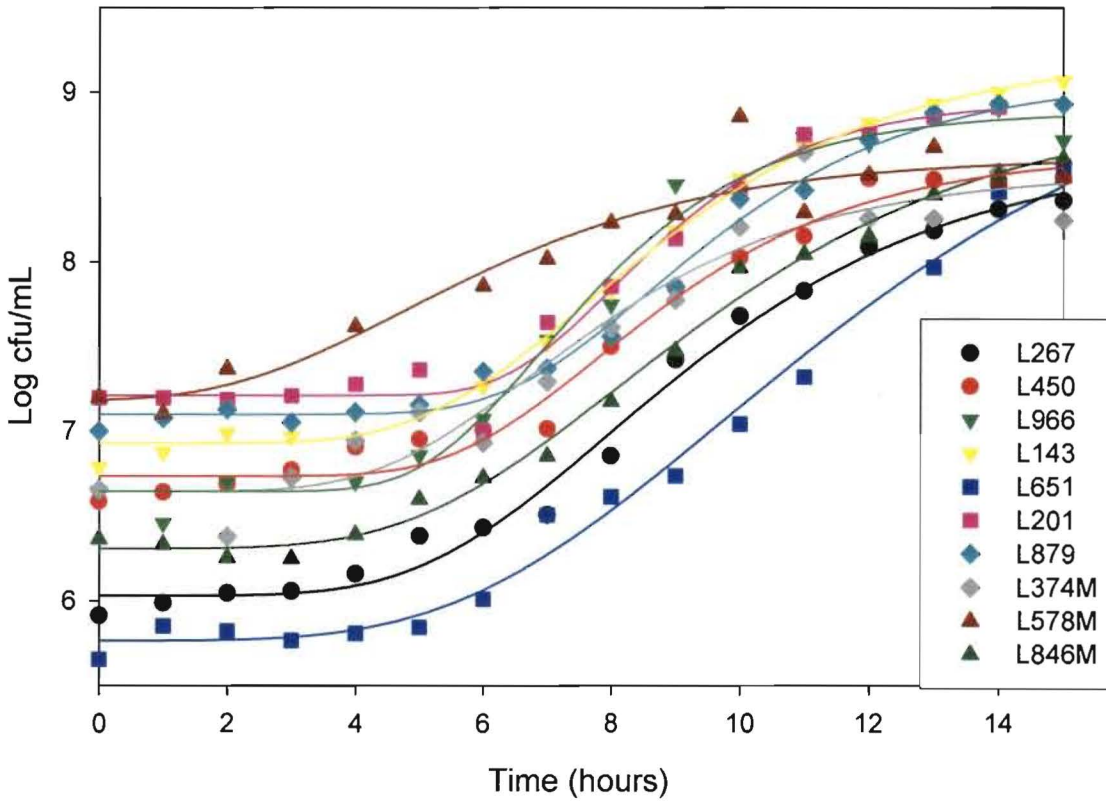

Figure 7: pH of ten *Lactobacillus helveticus* cultures in MRS broth.

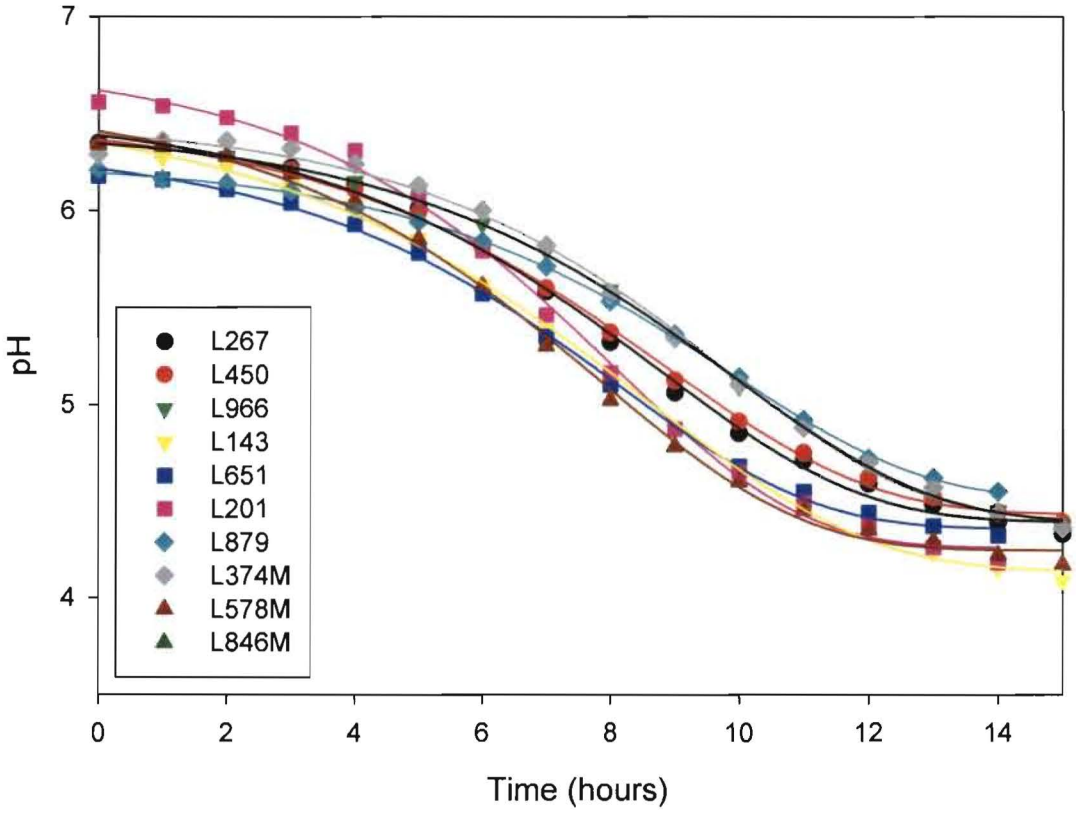

Figure 8: Absorbance (600 nm) of ten *Lactobacillus helveticus* cultures in MRS broth.

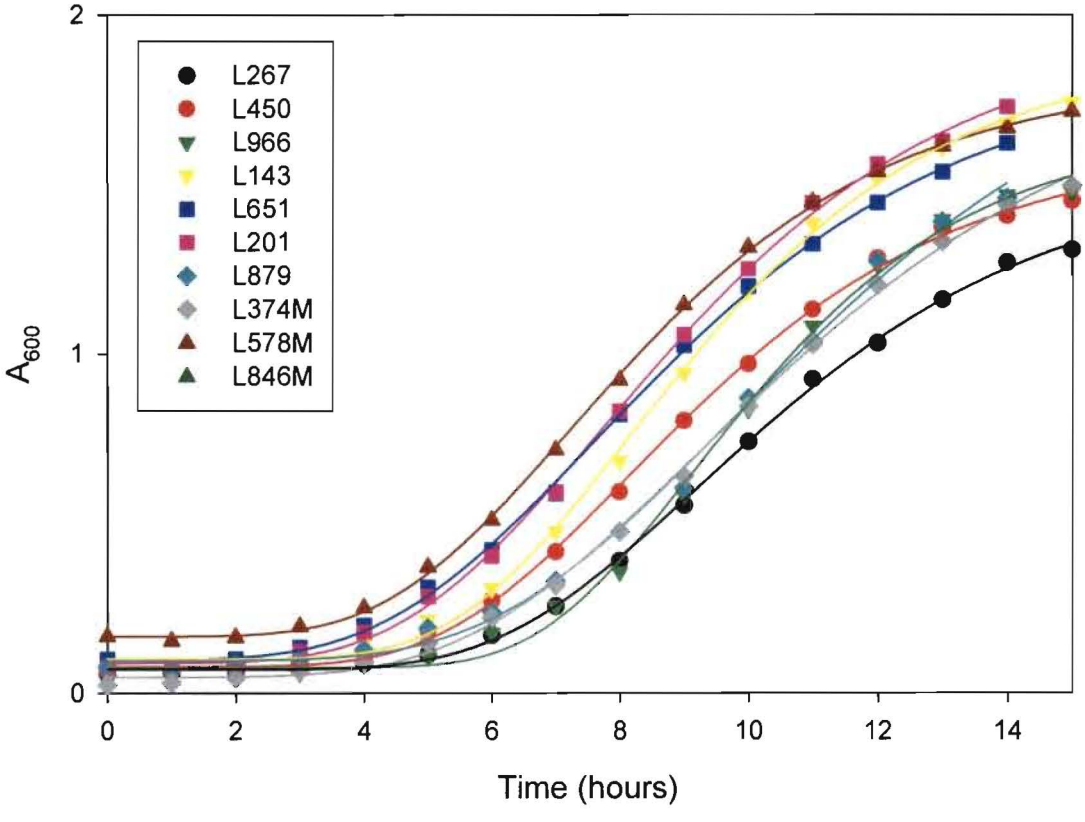

Figure 9: Growth of ten *Lactobacillus helveticus* strains in milk

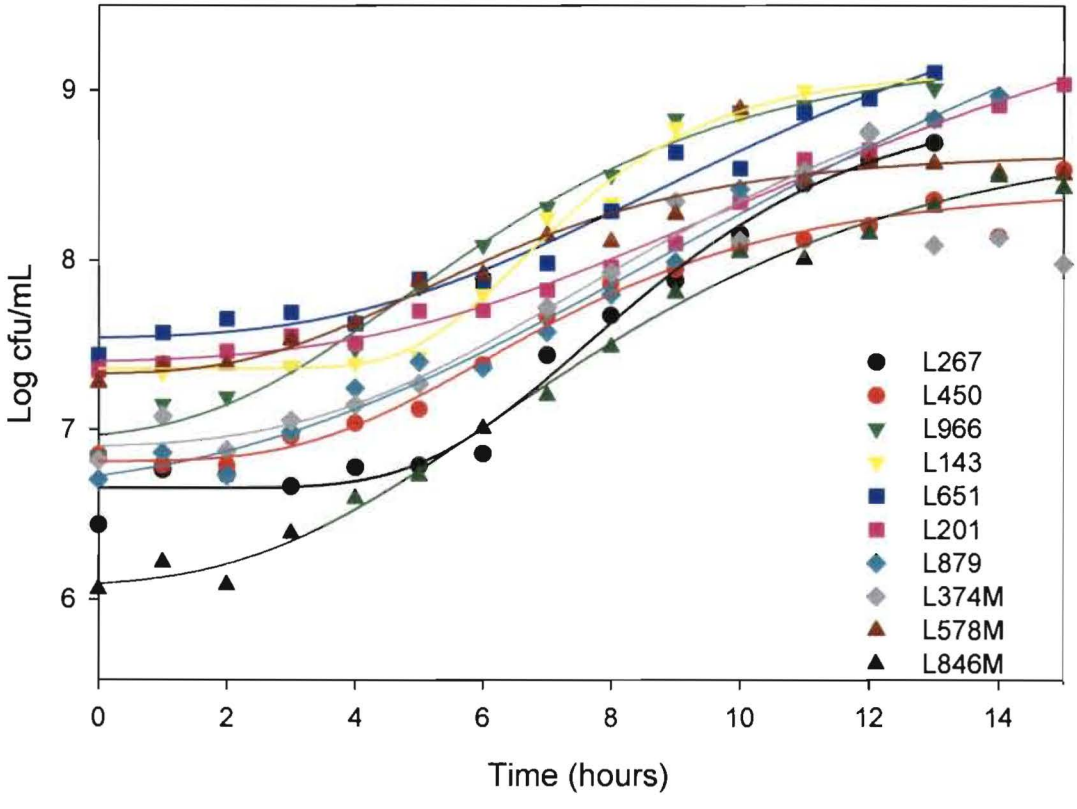

Figure 10: pH of ten *Lactobacillus helveticus* cultures in milk

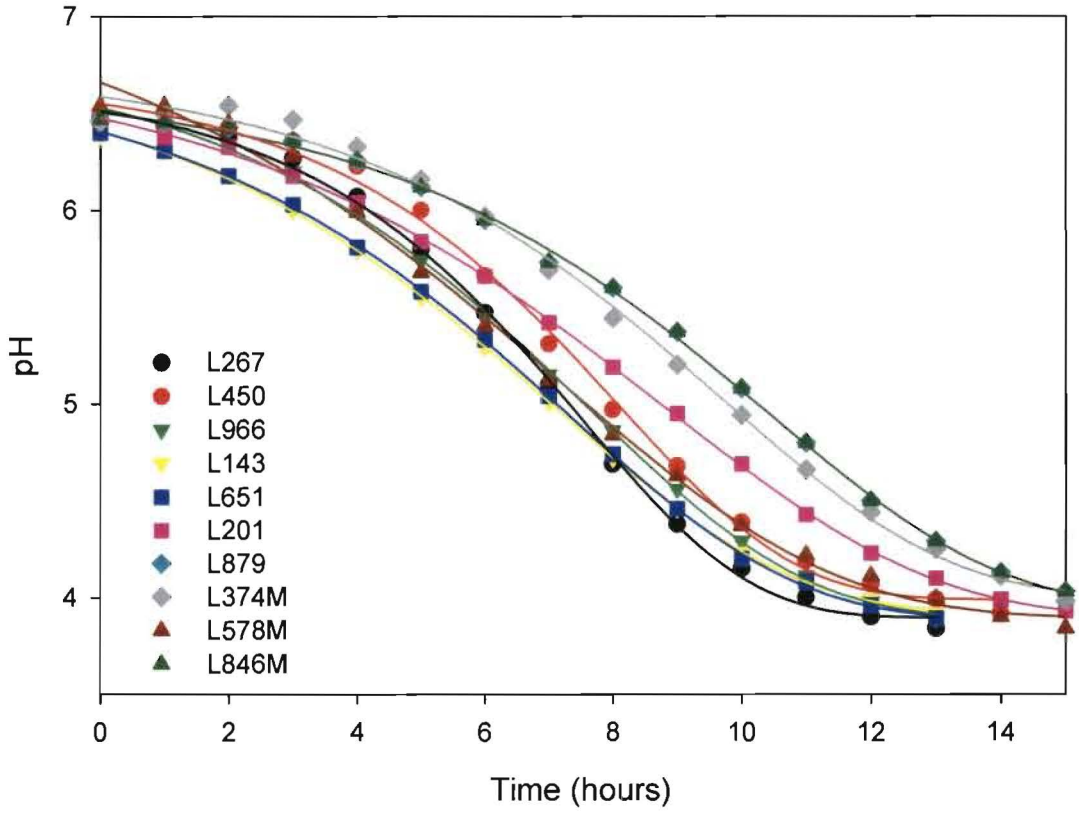

Figure 11: Growth Of six *Propionibacterium* strains in sodium lactate broth

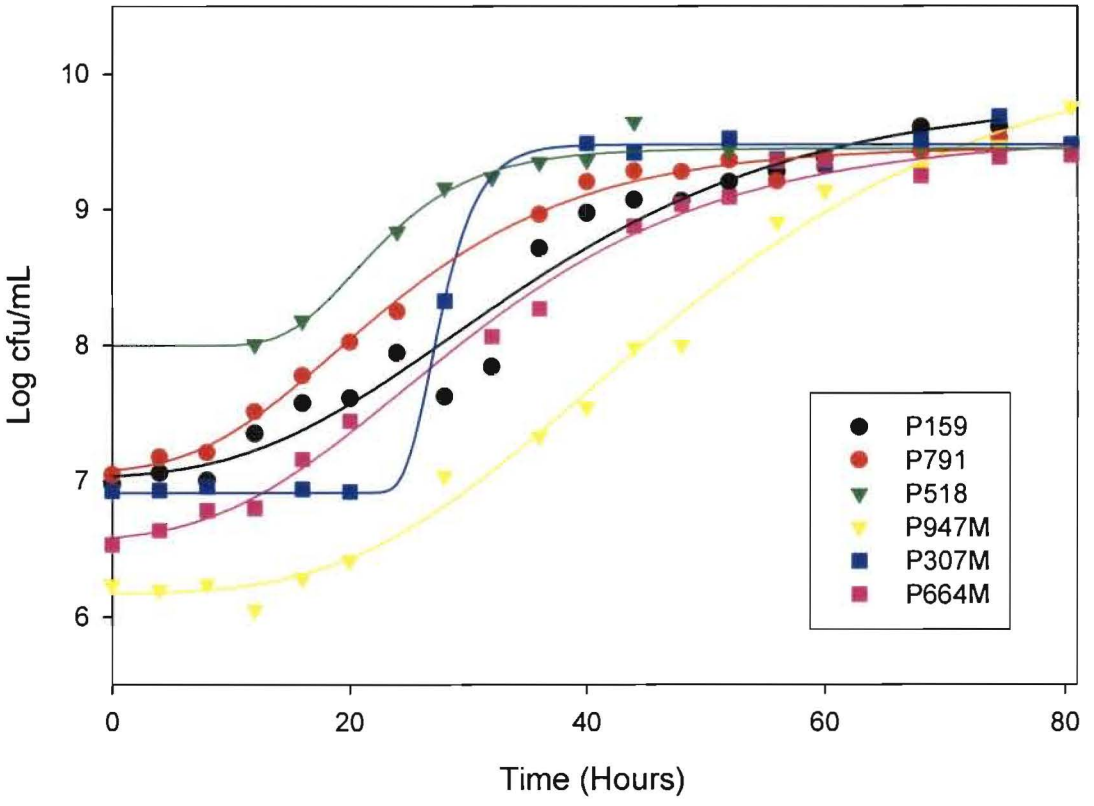

Figure 12: Absorbance of six *Propionibacterium* cultures in sodium lactate broth.

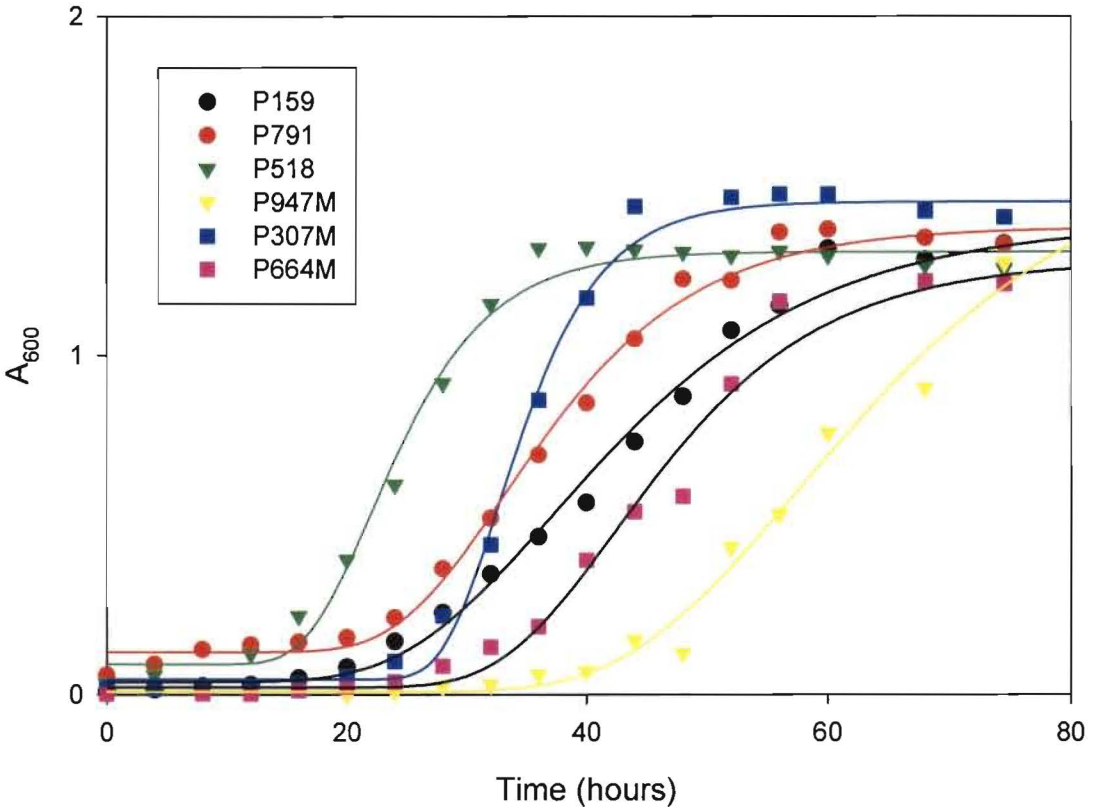

Figure 13: pH of six *Propionibacterium* cultures in sodium lactate broth

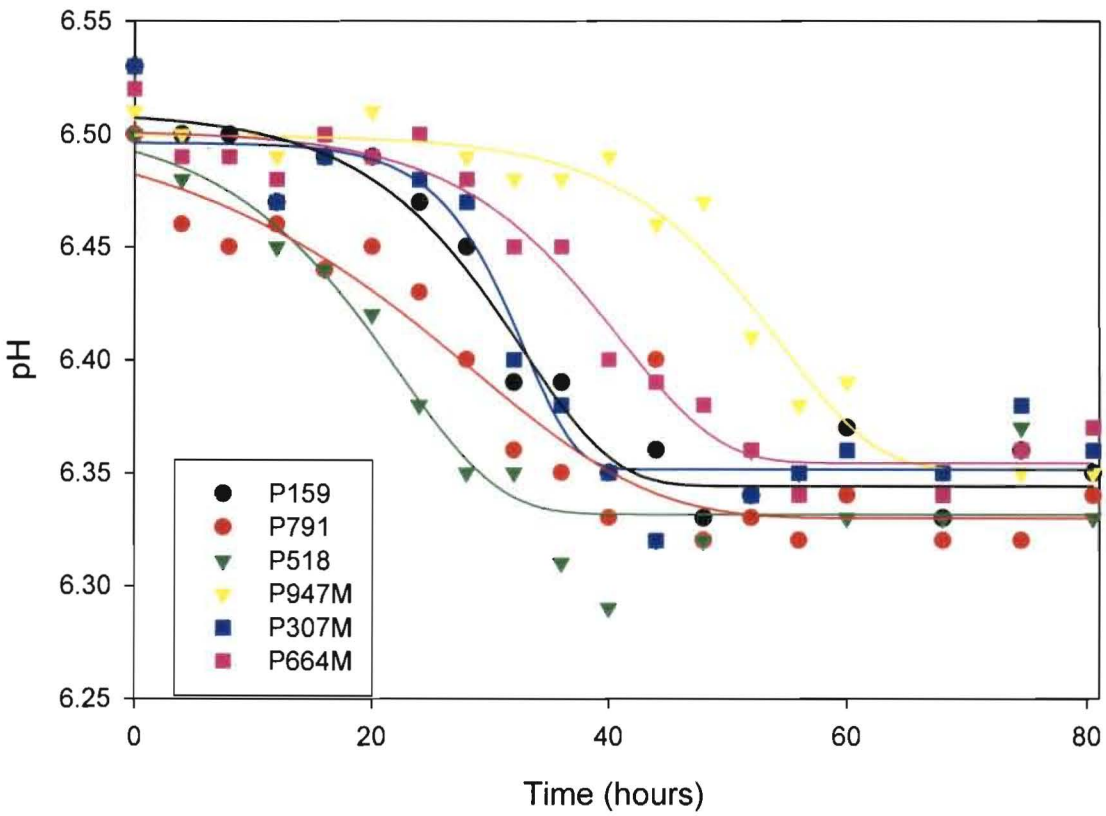

Figure 14: Gas production of six *Propionibacterium* cultures in sodium lactate broth.